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The Role of MTHFR C677T Polymorphism on Blood Homocysteine Concentration and the Effect of Both of this on Susceptibility to Stroke

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Abstract

Overproduction of homocysteine exerts toxic effect on endothelial linage of blood vessels supplying blood into the brain and developing condition of Stroke. Methylenetetrahydrofolatereductase (MTHFR) plays a central role in regulation of homocysteine concentration in the cell. The (677C>T) polymorphic variant of MTHFR gene is known to hyperhomocysteinemia and hens 677C<T MTHFR variant may be a factor for stroke susceptibility. This study investigated whether the genetic variability in the MTHFR gene is related with overproduction of homocysteine as well as susceptibility for Stroke in the central Indian population. A total of 100 medically certified Stroke patients (case) and 223 healthy subjects (control) were recruited from central India as a sample for this investigation. We found a significant (P<0.0001) deference in mean values of tHyc between case and control. 'T' allele was present in higher proportion in Stroke patients (P=0.06) as compared with Healthy control group. We concluded that the homozygous MTHFR 677T condition made moderate susceptibility for hyperhomocysteinemia and Stroke.

Keywords: Hyperhomocysteinemia; Stroke; Polymorphism; Endothelial dysfunction

Introduction

Stroke is leading cause of death among cardiovascular diseases and most prevalent factor for disability in world population [1]. Stroke arises in most of the chances by damaging internal endothelial lining of the blood vessels. The homocysteine exert its toxic effect on damaged endothelium by enhanced lipid peroxidation and generation of free radicals result into inflammation [2]. Due to developing inflammation, artery gets choked leading to partial to complete blockage of blood supply to the respective organ. An increased homocysteine in the blood is thus related with acute endothelial dysfunction [3]. The excess homocysteine is remethyleted into methionine and this step is catalyzed by Methionine synthase, which uses B12 as coenzyme and methylene-tetrahydrofolate (MTHF) as substrate. The formation of MTHF from tetrahydrofolate is catalyzed by Methylene-tetrahydrofolate reductase (MTHFR) [4]. The C677T mutation of the MTHFR gene, which leads to the synthesis of a thermolabile form of MTHFR that is responsible for 50% of the MTHFR activity [5].

Materials and Methods

Sample Collection

Patient Recruitment: Medically certified Hypertensive and Stroke patients were recruited from medicine department (OPD) of Shyam Shah medical college, Rewa, Madhya Pradesh, India. 100 Stroke patients were recruited for present investigation.

All the recruited patients were Central Indian origin mostly from Rewa, Jabalpur, Bhopal and Indore. The Stroke patients were recruited those who previously attacked by any type of Stroke.

Healthy Controls: 223 randomly selected healthy controls (HC) were enrolled in the study. The control group consisted of medical staff and healthy volunteers from Rewa, Jabalpur, Bhopal, Indore as well as individuals residing in central region of India. Hence, control group was drawn from same area with similar environmental and social factors with same mean age and sex ratio.

Sample Collection Strategy: Approximately 5 ml. of blood sample was collected in 0.5 M EDTA tubes from each Hypertensive and Stroke patients as well as from healthy controls. These samples were stored frozen at -80°C until DNA was extracted from them.

Sample Selection: Medically certified 100 Stroke patients and 223 healthy control were recruited from Central Indian population. 5 ml. of blood sample was collected in 0.5 M EDTA tubes from each patient as well as from healthy controls.

Homocysteine Analysis: Homocysteine analysis was done by autoanalyzer with the help of Kit (DIAZYME). The Diazyme Homocysteine Enzymatic Assay (by DIAZYME laboratories, catalogue no. DZ1 12 A-K) is based on a novel assay principle that assesses the co-substrate conversion product (a molecule that is not a substrate of the Hcy conversion enzyme. In this assay, oxidized Hcy is first reduced to free Hcy which then reacts with a co-substrate, S-adenosylo-methionine (SAM) catalyzed by a Hcy S-methyltransferase to form methionine (the Hcy conversion product of Hcy) and S-adenosylhomocysteine (SAH, the co-substrate conversion product). SAH is assessed by coupled enzyme reactions including SAH hydrolase, adenosine (Ado) deaminase and glutamate dehydrogenase, wherein SAH is hydrolyzed into adenosine (Ado) and Hcy by SAH hydrolase. The formed Hcy that is originated from the co-substrate SAM is cycled into the Hcy conversion reaction by Hcy S-methyltransferase. This forms a co-substrate conversion product based enzyme cycling reaction system with significant amplification of detection signals. The formed Ado is immediately hydrolyzed into inosine and ammonia which reacts with glutamate dehydrogenase with concomitant con-versions of NADH to NAD+. The concentration of Hcy in the sample is indirectly proportional to the amount of NADH converted to NAD+ (ΔA340nm).

DNA isolation and Quantification

Genomic DNA was extracted from whole blood by the modification of salting out procedure described by Miller and coworkers [6]. The integrity of high molecular weight DNA is an important factor, which should be considered during extraction steps. Integrity was checked by electrophoresis on 0.8%. The high molecular weight genomic DNA appeared as a single band near the well. DNA was quantified by measuring the optical density at 260nm. 5 µl of stock genomic DNA was taken and 995 µl of water was added (Dilution factor D.F. = 200), mixed well and OD was taken at 260 nm in a spectrophotometer (Systronic, India).

The Detection of MTHFR C677T Polymorphism

The MTHFR C677T polymorphism was sought using a PCR-RFLP method. The transition of C→T at the position 677 produces restriction site for HinfI, by which the polymorphic MTHFR gene and wild type gene can be identified after PCR amplification, restriction digestion and gel electrophoresis.

Primers: Primers were synthesized by Sigma Aldrich, India and amplification was carried out using MJ research thermolcycler those described by

Primer Sequence:

Sense:	5'-TGAAGGAGAAGGTGTCTGCGGGA-3'
Anti sense:	5'-AGGACGGTGCGGTGAGAGTG-3'

PCR Mix: 25 µl of each PCR reaction mixture contained 2-5µl template DNA (final concentration 100-200 ng/ µl), 2.5 µl of 10X *Taq* polymerase buffer (10 mM Tris HCl pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.005% Tween-20, 0.005% NP-40; final concentration 1X; Genetix Biotech Asia Pvt. Ltd., India), 1 µl of 10 mM dNTPs (Bangalore Genei, Bangalore, India), 1 µl of 10 pm/µl of forward and reverse primers specific for MTHFR gene, 0.3 µl of 5U/µl of *Taq* DNA polymerase (final concentration 1.5U; Bangalore Genei, Bangalore, India) and sterile water to set up the volume of reaction mixture to 25 µl.

Thermal Profile: Thermal profile used for the amplification of desired segment of gene was as follows: Initial denaturation at 94˚C for 2 min and 35 cycles of denaturation at 94˚C for 1 min, annealing at 58˚C for 1 min and extension at 72˚C for 1 min, followed by final extension at 72˚C for 10 min.

Restriction Digestion by HinfI: The digestion mixture contained 1 µl of HinfI (10 units/µl) (Roche Diagnostics, Meylan, France), 5 µl of the digestion control PCR product and 10 µl of the patient's PCR product in a final volume of 25 µl. It was incubated at 37°C for 4 h. DNA Fragment length analysis was done 10.2% polyacrylamide gel Electrophoresis. The Mutant (677TT) genotype cleaved into 175 and 23 bp, while wild (677CC) genotype of 198 bp intact without insertion.

Statistical Analysis: We analyzed data by Fischer's exact test, unpaired t-test, and Odds ratio with 95% confidence interval and interpret results for P value.

Results

The demographic parameters of study groups are presented in (Table-1) which shows there are insignificant changes in these parameters and individuals in both the groups are same except the disease.

In this study we found elevated Homocysteine concentration in patients than control with wild 'CC' genotype carrying individuals. The present study found 22.27± 5.89 µmol/L Homocysteine in 'CC' genotype carrying patients and 10.56 ± 2.24 µmol/L Homocysteine in 'CC' genotype carrying control (Table 2).

Table 1 Clinical features of Stroke Patients and Healthy Controls

Clinical features	*Stroke patients*	*Healthy controls*
Total number	100	223
Sex (Male: Female)	77:23	138:82
Mean BMI ± SD	27.66 ± 4.84	22.75 ± 4.52
Age (Years)		
Mean ± SD	52.79 ± 9.98	49.79 ± 12.66
Age range	40-76	25-78
Mean Homocysteine (µmol/L) Value ± SD	24.04 ± 6.01	11.17 ± 2.40

Table 2 The Analysis of differences in mean values of total Homocysteine in blood between case and control groups to find significant differences by t-test.

Genotype	tHyc		tHyc		t-test	95% CI
	N	µnol/L ± SD	N	µnol/L ± SD	(Bonfferoni correction)	
	(100)	Case	(223)	Control		
CC	66	22.27±5.89	184	10.56±2.24	P>0.0002	-12.72- 10.70
CT	28	26.93± 4.133	34	12.57±1.6	P>0.0002	-15.90-12.82
TT	06	29.95±13.18	05	18.66±1.63	P=0.182	24.83-22.48

This observation suggested that Homocysteine is significantly associated with disease. The present study also found that the elevation of Homocysteine is increasing with mutation. The heterozygous mutant 'CT' and homozygous mutant 'TT' is found to sharp homocysteine elevation in patients than control (Table 2). These values of Homocysteine between case and control are statistically examined for significant differences by nonparametric t-test and p values are corrected (adjusted) with Bonferroni Correction and results are presented in Table 2. This study found significant differences (P<0.0002) in Homocysteine level between case and control group carrying same genotypes (Figure 1).

These observations suggest the mutation in MTHFR is related with hyperhomocysteinemia. To observe role of mutation in MTHFR to susceptibility for stroke we analyzed the distribution of wild and mutant genotypes and allele between patients and control.

The distribution of genotypes and alleles are statistically examined by Fischer exact test for significant difference and the values are given in Table 3. The homozygous mutant MTHFR 'TT' genotype frequency was found higher in Stroke (6% vs 2.3%) then controls, and an odds ratio of 1.50 (95% CI, 0.44- 5.09) was found in Stroke. 'T' allele was present in higher proportion in Stroke (P<0.06) as compared with Healthy Control group. An odds ratio for 'T' allele were 1.64 (95% CI, 1.05- 2.5) indicated approximately double frequency of 'T' allele among Stroke patients (Table 2) whereas odds ratio for 'C' allele were 0.60 (95% CI, 0.39- 0.94) for same. The P values after Bonferroni Correction was not found significant for any genotype and any allele of MTHFR. These observations suggest there is no correlation between MTHFR mutation and Stroke susceptibility.

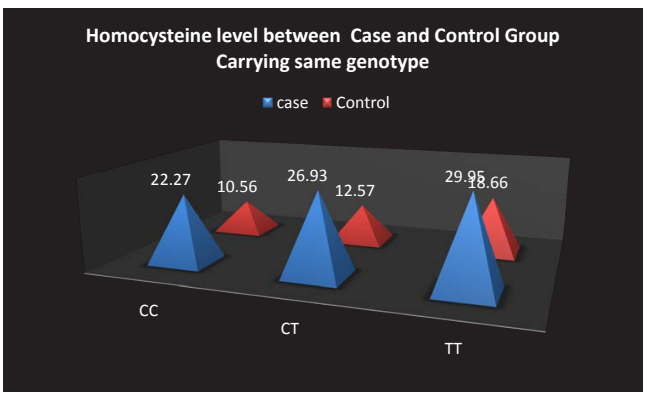

Figure1- Homocysteine level between case and control group carrying same Genotype

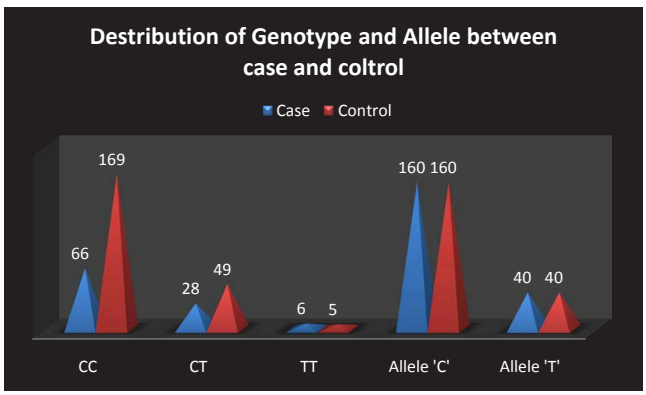

Figure 2 Distribution of genotype and allele between case and control

Table 3 The distribution of genotypes and alleles between Stroke patients and control are statistically examined by Fischer exact test for significant difference The P values after Bonferroni Correction.

Genotype and Allele	*Study Group* *Control*	*Patient*	*t-test (Bonfferoni correction)*	*OR*	*95% CI*
CC	169 (75.8%)	66 (66%)	0.158	0.6203	0.3706-1.038
CT	49 (21.9%)	28 (28%)	0.5196	1.381	0.8051-2.369
TT	05 (2.2%)	06 (06%)	1.095	1.506	0.4458-5.090
Allele 'C'	387 (86.85%)	160 (80%)	0.0664	0.6098	0.392-0.948
Allele 'T'	59 (13.21%)	40 (20%)	0.0664	1.640	1.05-2.55

Discussion

We have provided statistical evidence that the MTHFR 677C >T polymorphism has effects on blood concentration of Homocysteine in people of Central Indian origin. Homocysteine has long been recognized as a strong modifier of Stroke risk. Our findings provide a plausible genetic basis for the Homocysteine elevation in Stroke risk. The 677 Cytosine of MTHFR genes is replaced by Thiamine nucleotide leading to substitution of valine from alanine at the site of 222 in polypeptide chain responsible for thermolabile enzyme with reduced activity. The enzyme shows irresponsiveness to their substrate binding and reduces efficiency of remethylation of Homocysteine. Remethylation of Homocysteine is essential consumption pathway of Homocysteine by which Homocysteine is converted into methonine hence blood Homocysteine elevated due to MTHFR 677C >T polymorphism. It is observed that wild 'CC' gene was able to regulate the remethylation pathway whereas heterozygous mutant (CT) moderately regulate while homozygous mutant (TT) is fail to regulate remethylation of Homocysteine which lead to successive elevation of blood Homocysteine in case and control by increasing degree of mutation in MTHFR gene.

Our findings are also suggested that the Homocysteine elevation in blood is also influenced by factors (environmental and life style) other than MTHFR 677C >T polymorphism. These suggestions based on the finding of higher concentration of blood Homocysteine in wild genotype carrying stroke patients. The blood Homocysteine concentration was also increasing with increasing degree of mutation in both groups, hence the Homocysteine elevation is a complex physiological phenomenon influenced by genetic, environmental and life style factors. Present investigation on Homocysteine elevation with stroke susceptibility is found the Homocysteine elevation due to MTHFR 677C >T polymorphism is independent risk factor for Stroke.

The Polymorphic genes translated into a qualitatively changed MTHFR enzyme which disturbs the regulation of homocysteine metabolic pathway and produces toxic level of Homocysteine for endothelium. The common polymorphism in MTHFR gene is C677T which decreases the enzyme activity, thereby elevating homocysteine levels. Weisberg reported that heterozygous MTHFR C677T polymorphism decreases its activity by approximately 30%, whilst in homozygous individuals the activity decreases by about 60% [7].

This is first study from central Indian population in which we found hyperhomocysteinemia as an independent risk factor for stroke. The wild 'CC' genotype had a protective effect on hyperhomocysteinemia whereas heterozygous mutant 'CT' and homozygous mutant 'TT' genotype responsible for hyperhomocysteinemia. Among the deferent genotypes of MTHFR C677T alleles shown a weak but significant interaction with disease and found low P value for Stroke but strong significantly associated with hyperhomocysteinemia. These findings are consistent with Indian studies [8,9] but in Japanese population only women associated with disease susceptibility [10].

Several studies on North Indian population were found significant association of MTHFR genotype with hyperhomocysteinemia but not with Stroke [11]. A study from west Bengal has found no significant correlation between the studied factors (hyperhomocysteinemia, TT and CT genotypes) and single vs recurrent stroke [12]. South Indian population has shown slightly increased frequency of CT genotype which is significantly associated with hyperhomocysteinemia as well as myocardial infarction [13]. In eastern Indian population MTHFR genotype is not significantly associated especially with Stroke but found in positive correlation with hyperhomocysteinemia [14,15].

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Disclosure

The authors have no conflicts of interest to disclose in this work.

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